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Enzyme Catalysis

Are Environmentally Coupled Enzymatic Hydrogen Tunneling Reactions Influenced by Changes in Solution Viscosity?**

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The role and importance of protein dynamics in enzymatic reactions remains a key question in enzymology.[1–5] Of great current interest is whether enzymes have evolved to use quantum tunneling to the best advantage by, when necessary, coupling specific protein motions (vibrations) to the tunneling reaction coordinate. It is now widely accepted that H tunneling (proton, hydrogen, or hydride) occurs in enzyme-catalyzed reactions,[1,6–11] but the role of promoting motions in modulating the tunneling barrier remains contentious.[3] Experimental identification of coupled (promoting) motions is challenging, with experimental evidence for environmentally coupled H-tunneling reactions mainly inferred from the unusual temperature dependence of primary kinetic isotope effects (KIEs).[12–15] In addition to temperature,[12,13] there are other intensive (bulk) properties that, in principle, can be used to probe these reactions, including pressure[10,16] and solution viscosity.[17–21] We have demonstrated the utility of combining pressure and temperature to study environmentally coupled H tunneling in the reductive half-reaction (RHR) of morphinone reductase (MR), involving hydride tunneling from the coenzyme NADH to the enzyme-bound cofactor flavin mononucleotide (FMN).[10] Although changes in solution viscosity have been used to probe protein rearrangement after CO dissociation from myoglobin,[21] configurational and conformational gating of interprotein electron-transfer (ET) reactions,[17–20] and conformational gating of intraprotein ET,[22] studies of the dynamical influence of solvent viscosity on enzymatic H-tunneling reactions are less common. Moreover, there has been no rigorous quantitative analysis of these effects.

At a phenomenological level, we have shown that the magnitude and temperature dependence of the reaction rate and KIE measured for proton tunneling in the RHR of methylamine dehydrogenase (MADH) are unchanged following the addition of 30 % glycerol (viscosity increase ca. 2–3-fold) to the reaction solution.[12] Similarly, a decrease in KIE and increase in apparent enthalpy for the RHR of L-phenylalanine oxidase (PAO) upon the addition of 30 % glycerol has been reported.[23] Glycosylation of a protein surface can also affect the dynamic motion of a protein, and this approach has been used to study the rate of hydride transfer in glucose oxidase (GO)[24,25] by 1) using various glycoforms of the enzyme that differ in the extent of glycosylation[24] and 2) replacing the native polysaccharide with different polymeric forms of polyethylene glycol.[25] By both increasing and decreasing the apparent surface viscosity, decreases in the fitness of the enzyme were observed, reducing the Arrhenius pre-exponential ratio (A_D/A_T) for deuterium and tritium transfer away from unity.[24,25] GO, MADH, and PAO do not have strongly temperature-dependent KIEs, suggesting a minor role (if any) for fast, non-equilibrated promoting motions coupled to the H-transfer coordinate. Herein, we report a more systematic analysis of the effects of solution viscosity on the rate of H transfer and primary KIE for the RHR of MR. This is a well-characterized environmentally coupled H-tunneling reaction with a KIE that is strongly dependent on temperature, consistent with a dominant promoting motion coupled to the reaction chemistry.[9,10,26–28]

The rate of hydride transfer from NADH to FMN in MR can be measured directly using rapid-mixing (stopped-flow) methods, and the observed rate of flavin reduction k_{obs} (which is concomitant with H transfer) can be described by Equation (1), where k_{lim} is the pseudo-first-order rate of FMN

$$k_{obs} = k_{rev} + k_{lim}[\mathrm{NADH}]/(K_S + [\mathrm{NADH}]) \qquad (1)$$

reduction under saturation conditions (when $[\mathrm{NADH}] \gg K_S$) and k_{rev} is the rate of the reverse reaction ($k_{rev} \approx 0$ with MR). The effect of solution viscosity on the RHR of MR with NADH as the reductant was investigated first by measuring the NADH concentration dependence of the reaction in different concentrations (0–60 wt %) of glycerol (Figure 1). As these measurements were made using a rapid-mixing (stopped-flow) device, we were able to increase the viscosity by only approximately tenfold before mixing artifacts compromised further measurements. Over the viscosity range of about 0.9–9 cP (Table 1), we observed an approximately tenfold increase in K_S values but little change in k_{lim} (the chemical step). The second-order rate constant for NADH capture (k_{lim}/K_S; Table 1) is predicted from von Smoluchowski theory[29] to be inversely proportional to the bulk solution viscosity; a value of unity is expected for the slope of the

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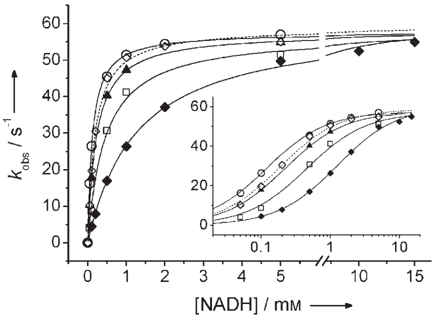

Figure 1. The effect of glycerol concentration on the saturation kinetics of flavin reduction by NADH in MR. The inset shows the same data on a log concentration scale. The data are fit to Equation (1), and the resulting k_{lim} and K_S values are given in Table 1. Conditions: 20 μM MR was mixed with NADH in 50 mM potassium phosphate, 2 mM 2-mercaptoethanol, pH 7, supplemented with 0 (○), 20 (▲), 40 (□), and 60 wt% (◆) glycerol at 25 °C. Also shown is the effect of 33 wt% sucrose (◇, dotted line).

Table 1: The effect of solution viscosity on the saturation kinetics of the reductive half reaction of MR with NADH at 25 °C.

η [cP]	[viscosogen] [wt%]	k_{lim} [s^{-1}]	K_S [μM]	k_{lim}/K_S [μM^{-1} s^{-1}]
0.90	0[a]	57.6 ± 1.1	119 ± 12	0.48 ± 0.06
1.55	20[a]	57.2 ± 1.1	203 ± 14	0.28 ± 0.03
3.19	40[a]	57.3 ± 1.8	454 ± 42	0.13 ± 0.02
3.2	33[b]	58.9 ± 1.2	187 ± 15	0.31 ± 0.03
8.82	60[a]	60.0 ± 0.7	1243 ± 54	0.05 ± 0.01

[a] Glycerol. [b] Sucrose.

normalized rate versus normalized viscosity, given in Equation (2), where η and η_0 are the solution viscosity in the presence and absence, respectively, of viscosogen.

$$m_{un} = \mathrm{d}([k_{lim}/K_S]_{\eta_0}/[k_{lim}/K_S]_{\eta})/\mathrm{d}(\eta_0/\eta) = 1 \qquad (2)$$

Initially, this appeared to be the case, as $m_{un} = 1.1 \pm 0.1$ (not shown). However, this trend appears to be viscosogen-dependent as, when k_{lim} and K_S were measured in 33% sucrose, the value of k_{lim}/K_S was found to be approximately threefold greater than that measured in 40% glycerol (Figure 1, Table 1), despite these two solutions having the same viscosity (see the Supporting Information). We have observed partial occupancy of glycerol in the active site of MR during X-ray crystallography studies,[41] and it is likely that this difference in k_{lim}/K_S values is due to weak competitive inhibition of MR by glycerol. Indeed, the steady-state reaction of MR with cyclohexenone is competitively inhibited by glycerol with a K_I value of 9–10 M (60–65 wt%) glycerol (see the Supporting Information). Despite these complications, the rate of k_{lim}/K_S measured in 33% sucrose is measurably slower than that in buffer (Table 1), and $m_{un} \approx 0.4$, such that NADH capture is clearly viscosity-dependent. Thus, whilst NADH binding (k_{lim}/K_S) is dependent on solution viscosity, the rate of H transfer (k_{lim})—the chemical step—is not.

Kramers[30] was the first to develop a theoretical treatment of the dynamical influence of solvent viscosity on unimolecular reactions such as the pseudo-first-order FMN reduction/hydride transfer step during the RHR of MR. In the Kramers model, the rate constant is related to the solvent viscosity through a friction coefficient, and the relationship between rate constant and viscosity is more complex than that of a bimolecular reaction. If it is reasonably assumed that the solvent molecules are much smaller than the protein or enzyme under study, the effect of viscosity on the observed rate of a reaction can be described in combination with the Eyring equation according to Ansari et al. [Eq. (3), where σ,

$$k_{obs} = \frac{k_B T}{h}\left(\frac{1+\sigma}{\eta+\sigma}\right)\exp\left(\frac{\Delta S^{+}}{R}\right)\exp\left(\frac{-\Delta H^{+}}{RT}\right) \qquad (3)$$

in units of viscosity, is the contribution of the protein friction to the total friction of the system].[18,21] As ΔH^{+} and ΔS^{+} do not appear to be significantly viscosity-dependent,[12,18] their values can be determined independently from the temperature dependence of the reaction.[18] The limiting rate of hydride transfer in the RHR of MR was measured with both NADH and (*R*)-[4-^{2}H]-NADH between approximately 0.9 and 9 cP with 0–60 wt% glycerol, and in both cases, the observed pseudo-first-order rate constants were, within error, independent of viscosity (Figure 2). Typical values for σ [Eq. (3)] measured for conformationally coupled reactions in proteins are typically 0–10 cP.[18,21,31,32] We have modeled the expected viscosity dependence [Eq. (3)] for various values of σ between 0 and 100 cP with $\Delta G^{+} = \Delta H^{+} - T\Delta S^{+} = 63.1$ and 67.2 kJ mol^{-1} for H and D transfer, respectively (Figure 2). These ΔG^{+} values are in good agreement with the ΔH^{+} and ΔS^{+} values determined previously from the temperature dependences of these reactions.[9,27] From the modeling in Figure 2, it would appear that σ must be greater than approximately 50 cP for both H and D transfer in MR—a value much larger than reported values for gated ET reactions. Additionally, Equation (3) predicts that, whilst the rate of a reaction may be strongly dependent on viscosity, the KIE should be independent of viscosity. This follows because σ is related to the entire substrate–enzyme complex,

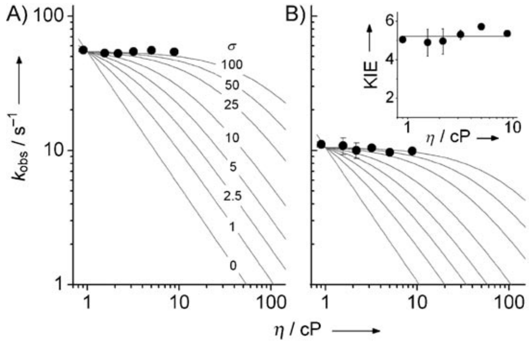

Figure 2. The effect of viscosity on the maximal rates of flavin reduction by NADH (A) and (*R*)-[4-^{2}H]-NADH (B). The inset shows the viscosity independence of the subsequent KIE. The rate-constant data are modeled (gray lines) according to Equation (3) for increasing values of σ. See text for details. Conditions: 20 μM MR was mixed with 5 mM (0–40% glycerol), 10 mM (50% glycerol), or 15 mM (60% glycerol) NADH or NAD^{2}H (final concentrations) in 50 mM potassium phosphate, 2 mM 2-mercaptoethanol, pH 7.0, supplemented with 0–60 wt% glycerol at 25 °C.

which should essentially be isotope-independent. This was found to be the case, and the viscosity independence of the primary KIE is shown in the inset to Figure 2.

Increasing solvent viscosity is thought to decrease the specific volume and the adiabatic compressibility of the protein interior.[33] It is also thought that viscosity and hydrostatic pressure may act on proteins similarly by decreasing the internal free volume and perhaps increasing protein rigidity.[34] We have recently measured the combined temperature and pressure dependence of the H-transfer step during the RHR of MR with NADH[10] and found that both the observed rate of H transfer and the primary KIE increased significantly at elevated pressure. This finding suggests that either the addition of 60 % glycerol has less effect on the free volume and rigidity of the MR–NADH complex than approximately 100 bar pressure (the increase in pressure required to measure a significant change in the rate of H transfer) or that viscosity and hydrostatic pressure do not act on MR in a similar way. To our knowledge, this is the first direct comparison of these effects on an enzymatic chemical step.

The magnitude of the viscosity dependence of the rate of interprotein[18–20,31] and intraprotein[22] ET reactions have been shown to be similar, despite the mechanisms of gating in these reactions being quite different (e.g. configurational rearrangement of the protein–protein interface vs. side-chain rearrangement). Why then have we measured no viscosity dependence of the H transfer during the RHR of MR, despite clear evidence that in this reaction, the transfer of the hydride is strongly coupled to (and gated by) the protein environment?[9,10,26–28] It is possible that Equation (3) is simply not valid for H-tunneling reactions. However, we feel that the more likely explanation for this discrepancy is that the mechanism of gating, at least in MR, differs significantly from that of the gated ET reactions studied. A network of long-range coupled motions has been proposed to drive the H-tunneling events in the enzymes liver alcohol dehydrogenase (LADH)[35,36] and dihydrofolate reductase (DHFR).[37] It is quite likely that the rate of the H-tunneling reactions in these enzymes will be viscosity-dependent, as these long-range motions are, presumably, quite similar to the conformational gating prevalent in many gated protein ET reactions. However, in some enzymatic H-tunneling reactions, most notably in aromatic amine dehydrogenase (AADH), the H transfer is assisted by a promoting motion,[38] yet this motion is not coupled to a network of long-range motions.[8] We feel that the most likely explanation for the insensitivity of the MR reaction to changes in solution viscosity is that the H-tunneling reaction in this enzyme, like in AADH, is not coupled to a network of long-range motions.

In summary, we have demonstrated, to our knowledge for the first time, that stopped-flow spectroscopy allows the measurement and deconvolution of the solution viscosity dependence of substrate capture and the subsequent chemical step in an enzyme. This is despite the limitations of the stopped-flow method for studying viscosity-dependent reactions attributed to inherent mixing problems at $\eta > 10$ cP. We have shown that substrate capture is viscosity-dependent, while the following H-transfer step—a well-characterized environmentally coupled quantum tunneling reaction[9,10,27]—is not. This finding is rationalized in terms of a lack of a network of long-range motions coupled to the H-transfer reaction in MR, similar to the case for AADH.[8,38] Finally, it has been recently suggested that coupled motions may not be catalytically important in DHFR.[39] We then suggest that solution viscosity studies may be a useful experimental method of identifying long-range motions and posit that if indeed the H-tunneling events in DHFR[37] and also in LADH[35,36] are coupled to long-range motions, then the rate of these reactions may well be sensitive to changes in solution viscosity.

Experimental Section

All materials were obtained from Sigma–Aldrich (St. Louis, MO), except NADH (Melford Laboratories, Chelsworth, U.K.) and [$^2H_6$]ethanol (Cambridge Isotope Laboratories, Andover, MA). MR was purified as described previously,[26,27,40] and (*R*)-[4-^{2}H]-NADH was prepared and characterized as described previously.[9,27] All experiments were performed in 50 mM potassium phosphate, 2 mM 2-mercaptoethanol, pH 7.0. Glycerol or sucrose solutions were prepared by weight, and the estimation of their solution viscosity is described in the Supporting Information. Rapid reaction kinetic experiments were performed using an Applied Photophysics SX.18MV-R stopped-flow spectrophotometer contained in an anaerobic glove box as described previously,[9] and spectral changes accompanying FMN reduction were monitored at 464 nm.[9,26,27]

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